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Use of hexose transport mutants to examine the expression and properties of the rat myoblast GLUT 1 transport process

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Abstract

Rat L6 myoblasts were recently shown to possess the GLUT 1, 3 and 4 transporters, and not the GLUT 2 isoform [1]. This investigation examined the expression and properties of the GLUT 1 isoform. GLUT 1 transcript level was significantly reduced in cells grown at high densities and during myogenic differentiation. A comparison of the GLUT 1 and 4 transcript levels in myogenesis-competent and impaired cells revealed an inverse relationship between these two isoforms. This relationship was confirmed by studies using two independent spontaneous GLUT 3^-GLUT 4^- mutants, M1 and M3. These mutants possessed very high level of the GLUT 1 isoform, but negligible amount of the GLUT 3 and 4 isoforms. GLUT 1 expression was also subject to positive regulation. Glucose starvation was found to increase not only the levels of the GLUT 1 transcript and transporter, but also the intrinsic activity of the GLUT 1 transporter. Studies with M1 and M3 mutants revealed that the GLUT 1 transporter was not functional in glucose-grown cells, even though it was present at a very high level in the plasma membrane. This transporter became functional when cells were starved for glucose. The functional GLUT 1 transporter had an apparent K_m value of around 0.9 mM, and was sensitive to cytochalasin B, phloretin, phlorizin and pCMBS.

Keywords: Glucose transporter; Transport mutant; Glucose starvation; Myogenic differentiation; Regulation; (Myoblast)

1. Introduction

Considerable effort has been focused in recent years on the regulatory mechanisms of glucose transport in eukaryotic cells. The uptake of glucose and its analogues is facilitated by a group of highly conserved glucose transporters (GLUT) [2–7]. Different mechanisms have been shown to regulate glucose transport activities. These include: (i) altered synthesis of the GLUT isoforms brought about by changing the transcription rates and/or stability of the mRNAs [2,8–11]; (ii) recruitment of glucose transporters from intracellular storage sites to the plasma membrane [6,12]; and (iii) modulation of the intrinsic activity of glucose transporter [3,6,13–20]. GLUT isoforms in the same cell are found to respond differently to metabolic and physiological changes, or upon exposure to different hormones or growth factors [1,21,22]. For example, the expression of GLUT 1 and 4 differs considerably in insulin-treated or glucose-starved cells [22], as well as at different stages of development [23–26].

An examination of the regulation and properties of a specific GLUT isoform is complicated by the presence of other GLUT isoforms in the same cell. For example, at least two glucose transport systems exist in chicken embryo fibroblasts, rat adipocytes, rat and human myoblasts, human skin fibroblasts and mouse 3T3 cells [22,27–34]. Even though the GLUT transcript and transporter levels can be determined, the similarities in their transport properties make it difficult to assess the functional state and kinetic property of a specific GLUT transporter.

One recent approach to examine the properties of individual GLUT isoforms is to express these isoforms in *Xenopus* oocytes [35–37]. While these studies are invaluable in determining the identity and general nature of the GLUT isoforms, they cannot provide information on the in situ functional states of the GLUT transporter under different metabolic and developmental conditions. In view of the

Abbreviations: BrdUrd, 5-bromo-2′-deoxyuridine; CB, cytochalasin B; dGlc, 2-deoxy-D-glucose; GLUT, glucose transporter; HAHT, high affinity hexose transport system; LAHT, low affinity hexose transport system; MeGlc, 3-*O*-methylglucose; PBS, phosphate-buffered saline; pCMBS, *p*-chloromercuribenzenesulfonic acid; SDS, sodium dodecyl sulfate.

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expression vectors used, this approach will not reveal how the expression of one GLUT isoform can be regulated by the presence of other GLUT isoforms. Another approach is to examine the properties of cell lines that possess only one GLUT isoform. For example, both Chinese Hamster Ovary (CHO) cells and mouse 3T3-L1 fibroblasts are found to possess only the GLUT 1 isoform [3]. However, these cells cannot be used to examine the influence of other GLUT isoforms on the expression of the GLUT 1 isoform.

Since most eukaryotic cells contain two or more GLUT isoforms, mutants possessing only one GLUT isoform can be used to examine the influence of other GLUT isoforms. A comparison of the mutant and parental properties will reveal not only the role(s) played by other GLUT isoforms, but also the in situ regulation of this particular GLUT isoform. More importantly, these mutants will allow an examination of the intrinsic property of a particular GLUT transporter without interference by other isoforms. The rat L6 myoblast system is uniquely suited for these studies. These cells possess the GLUT 1, 3 and 4 isoforms, but not the GLUT 2 isoform [1]. The glucose transport property of L6 myoblast in whole cells and in plasma membrane vesicles have been characterized by kinetic analysis, cytochalasin B (CB) binding and photolabelling studies, and by inhibitor studies [31,38–42]. Transport, and not substrate phosphorylation, is the rate-limiting step in the uptake of dGlc by rat myoblasts, even at very high substrate concentrations [31]. A high (HAHT) and a low affinity (LAHT) hexose transport process, as well as at least two CB binding sites have been detected in undifferentiated myoblasts. These two transport processes are regulated differently during metabolic and developmental changes [15,43,44]. Rat myoblast mutants defective in the GLUT 3 and/or the GLUT 4 isoforms have been isolated and characterized [1,44,45]. These mutants are invaluable in identifying the transcripts and transporters for various glucose transport processes; the GLUT 3 and 4 isoforms are found to be associated with the HAHT and LAHT, respectively. Studies with two independent spontaneous GLUT 3^-GLUT 4^- mutants indicated that the GLUT 1 isoform was the only GLUT isoform present in these cells [1]. The present investigation utilized these mutants to examine the in situ expression of the GLUT 1 isoform and its intrinsic activity under different growth conditions.

2. Methods and materials

2.1. Cell lines and culture media

Rat L6 skeletal myoblast was originally obtained from Yaffe [46]. Strain D23 is a spontaneous GLUT 3^-GLUT 4^+ mutant isolated from rat L6 myoblast [45]. Mutants M1 and M3 are two independent spontaneous GLUT 3^-GLUT 4^- mutants selected from D23 myoblast [1]. All cell lines were maintained in Alpha medium (Flow Laboratories) supplemented with 25 mM D-glucose, 10% (v/v) horse serum (Flow Laboratories) and gentamycin (72 μg/ml; Life Technologies) [47]. Cells cultured in this medium were referred to as glucose-grown cells. The glucose starvation medium was Alpha medium prepared without D-glucose and was supplemented with 25 mM fructose and 10% (v/v) dialysed horse serum.

2.2. Transport studies

Transport studies were carried out using six-well (35 × 15 mm) Falcon plates [15]. Cells were routinely plated at a density of $1.5 \cdot 10^5$ cells/well. After removal of medium, each well was washed with 10 ml of phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$), and replaced with 900 μl of the uptake buffer (PBS containing 1 mg/ml bovine serum albumin). Transport studies were carried out at 23° C and were initiated by adding 100 μl of radioactive substrate to the desired final concentration. At appropriate times, uptake was terminated by rapidly washing the cells twice (less than 15 s) with 10 ml of ice-cold PBS. In the case of MeGlc uptake, washes were carried out with cold PBS containing 1 mM mercuric chloride. Transport studies were carried out using the same range of substrate concentrations as in determining the GLUT 3 and 4 transport kinetics [31]. The initial rates of uptake were calculated from the amount of substrate taken up at 15, 30, 45 and 60 s. after addition of radioactive substrate. Cells were solubilized by adding 1 ml of 0.1% Triton X-100, and 0.8 ml aliquot was counted in 10 ml of scintillation fluid. Cells from two wells on each plate were detached with 0.1% trypsin, and the cell number was determined using a Coulter counter. Under our experimental conditions, the uptake of dGlc and MeGlc was linear with time, and over 95% of the dGlc taken up was phosphorylated [31]. Even at a concentration of 10 mM, transport and not phosphorylation is the rate-limiting step in dGlc uptake. Transport studies were carried out in duplicate and each experiment was repeated twice. Data were analyzed by a linear least squares regression fit program (SlideWrite Plus, Advanced Graphics Software, Sunnyvale, CA), and by a non-linear regression data analysis program (Enzfitter program, Biosoft, Cambridge, UK).

2.3. Isolation of mRNA from rat myoblasts

Poly(A)$^+$ RNA was isolated directly from rat myoblasts using the FastTrack™ mRNA isolation kit (Invitrogen, San Diego, CA), as previously described [1].

2.4. Preparation of cDNA probes

Plasmids harbouring GLUT cDNAs were purchased from the Repository of Human DNA Probes and Libraries,

American Type Culture Collection, Rockville, MD. The human GLUT 1 cDNA was originally provided by B. Thorens, Whitehead Institute for Biomedical Research, Cambridge, MA [48], whereas the human GLUT 3 cDNA was obtained from G. Bell, Howard Hughes Medical Institute, University of Chicago [49]. The rat GLUT 4 cDNA was a generous gift from M. Birnbaum, Harvard Medical School, Boston, MA [50]. β_2-Microglobulin cDNA was originally obtained from F. Daniel, Pasteur Institute, Paris, France [51]. Plasmid DNA was isolated by the alkaline lysis method [52]. Plasmid cDNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1); and once with chloroform/isoamyl alcohol (24:1). Different restriction enzymes were used for isolating cDNA inserts: *Bam*HI for GLUT 1, *Sal*I for GLUT 3, and *Eco*RI for GLUT 4. Plasmid DNA was incubated with the appropriate restriction enzyme for 2 h at 37° C. After electrophoresis through a 0.9% agarose gel and purification using the Gene Clean Kit (Bio/Can Scientific), the insert was labelled with ^{32}P using the Prime-a-Gene kit (Promega).

2.5. Northern gel blotting studies

For RNA blotting, 2 μg of poly(A)$^+$ RNA were denatured with formaldehyde [53]. An RNA ladder (BRL) was used as the standard molecular weight marker. After separation by electrophoresis through an 1.2% formaldehyde agarose gel, the RNA was transferred to a Biotrans nylon membrane (ICN Biochemicals) by a vacuum blotting system (VacuGene^{TM} XL, Pharmacia). The nylon membrane was then UV irradiated for 30 s at $1.2 \cdot 10^6$ μJ with the Stratalinker^{TM} UV Crosslinker 1800 (Stratagene). The blotted and fixed membrane was prehybridized at 42° C for 2–3 h in a solution containing 50% formamide, 5 × SSC, 2 × Denhardt's solution, 20 mM sodium phosphate buffer (pH 6.5), 0.1% sodium dodecyl sulfate (SDS), and 100 μg/ml denatured herring sperm DNA [52]. Hybridization with the ^{32}P-labelled cDNA probe was carried out at 42° C overnight in the prehybridization solution containing 10% dextran sulfate, and $1 \cdot 10^6$ cpm/ml of the ^{32}P-labelled insert. The blot was then washed with 0.1 × SSC and 0.1% SDS first at 23° C for 15 min and then at 55° C for 20–30 min; after which it was exposed to Kodak XAR-5 film at −70° C for 20 h. Bands in the autoradiogram were quantified using the JAVA Video Analysis Software, Jandel Scientific. All experiments were repeated at least twice with two different RNA preparations.

2.6. Purification of plasma membrane and microsome

Plasma membranes and microsomes were prepared from myoblasts as previously described [38,42,54]. Unlike most conventional methods [19,55,56], the crude plasma membrane obtained from differential centrifugation was further fractionated by centrifugation through a discontinuous sucrose gradient, consisting of 24, 29, 35 and 45% (w/w) sucrose. The plasma membrane was recovered in the 24% fraction and in the interface between the 24% and 29% fractions. Only 5% of the proteins loaded onto the sucrose gradient was recovered in these two fractions [38]. These two fractions were highly enriched in the plasma membrane marker enzyme, the ouabain-sensitive Na^+/K^+-ATPase, and were devoid of marker enzymes for mitochondria (NADH-dependent cytochrome-*c* reductase), microsomes (succinate-dependent cytochrome-*c* reductase), and cytoplasmic contents (lactate dehydrogenase and hexokinase).

2.7. Immunoblotting studies with membrane preparations

Membrane preparations were dissolved in the SDS-sample buffer at room temperature, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the discontinuous buffer system of Laemmli [57]. After electrophoresis, proteins were electrophoretically transferred from the gel onto nitrocellulose membranes using the transfer buffer (25 mM Tris, 192 mM glycine (pH 8.3), 20% methanol, and 0.1% SDS) at 55 V for 2 h [58]. Non-specific protein binding was minimized by incubating with 20% fetal calf serum for at least 5 h at 23° C in a Tris buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 0.05% Tween 20. The membrane was washed once with the same Tris buffer, and was then incubated with anti-rat GLUT 1, anti-mouse GLUT 3 or anti-rat GLUT 4 antibodies (East Acres Biologicals, Southbridge, MA) for 14 h at 23° C. After washing with the same Tris buffer three times (5–10 min for each wash), the membrane was incubated with ^{125}I-protein A for 4 h. The unbound ^{125}I-protein A was removed by washing three times with the Tris buffer, blots were dried and exposed to Kodak X-Omat film at −86° C. After identifying the GLUT 1 transporter band on the autoradiogram, the corresponding spot on the nitrocellulose paper was cut and counted in a γ-counter. Specific binding was determined by subtracting background radioactivity present on a size-matched strip of the filter paper cut out from an area next to the GLUT 1 band on the same lane. Experiments were repeated at least twice.

2.8. Determination of fusion index

On different days after subculturing, cells were treated with 1 mM $ZnSO_4$ in 20% DMSO to swell the nuclei and fixed in 2.5% glutaraldehyde in PBS, and then stained with 6% Giemsa [43]. Fusion index was calculated as the percentage of total nuclei present in myotubes [44,59]. Only structures containing at least three nuclei were considered as myotubes. Usually each field contained at least 50 nuclei. Ten fields were counted for each set of determinations.

2.9. Materials

2-Deoxy-D-[1,2-^{3}H]glucose (50 Ci/mmol) and 3-*O*-[*methyl*-^{3}H]methyl-D-glucose (50 Ci/mmol) were purchased from ICN Biochemicals. [α-^{32}P]dCTP and ^{125}I-labeled protein A were from Amersham Canada. Restriction enzymes, Prime-a-Gene kit, and molecular weight markers were purchased from Promega. Cytochalasin B, pCMBS, phloretin, phlorizin and 5-bromo-2′-deoxyuridine were purchased from Sigma. All other chemicals were purchased from commercial sources and were of the highest available purity.

3. Results

3.1. Negative regulation of GLUT 1 expression in rat myoblasts

Poly(A)$^+$ RNAs from myogenesis-competent L6 cells were used to examine the expression of the GLUT 1 isoform during myogenesis. A 10% drop of the GLUT 1 transcript level was observed between days 3 and 4; whereas a 40% drop occurred between days 4 and 5. Thus, dramatic reduction of the GLUT 1 transcript was observed only upon appearance of multinucleated myotubes (i.e., after day 4) (Fig. 1). This was distinct from the uniform rate of decline observed for the GLUT 3 transcript from day 2 to day 6 [1].

To determine the relationship between the decline in the GLUT 1 transcript and myogenesis, poly(A)$^+$ RNAs were prepared from L6 cells treated with myogenesis inhibitors, such as 5′-bromo-2-deoxyuridine or phloretin [44,60]. The GLUT 1 transcript level was reduced at a much slower rate in myogenesis-impaired cells than that in myogenesis-competent cells (Fig. 1). When compared with day 2 cultures, the GLUT 1 transcript was reduced by 30% and 60% in day 5 culture of the myogenesis-impaired and competent cells, respectively. Thus, GLUT 1 expression was affected, directly or indirectly, by events associated with myogenesis.

A comparison of the GLUT transcript levels during myogenesis revealed that multinucleated myotubes (day 6 culture) possessed about 25% (Fig. 1) and 7500% [1] of the day 2 GLUT 1 and 4 transcript levels, respectively. Such an inverse relationship between GLUT 1 and 4 expression was also observed in myogenesis-impaired cells. Day 5 culture of phloretin-treated cells harboured 70% and 200% of the day 2 GLUT 1 and GLUT 4 transcript levels, respectively (Fig. 1, [1]). More interestingly, while the GLUT 4 transcript level was elevated at an uniform rate during myogenesis [1], dramatic decreases in the GLUT 1 transcript (between day 4 and 5) (Fig. 1) occurred only after a 4-fold increase in the GLUT 4 transcript (day 3.5) [1]. Thus, similar to adipose tissues [61], increases in the GLUT 4 transcript preceded the major decline of the

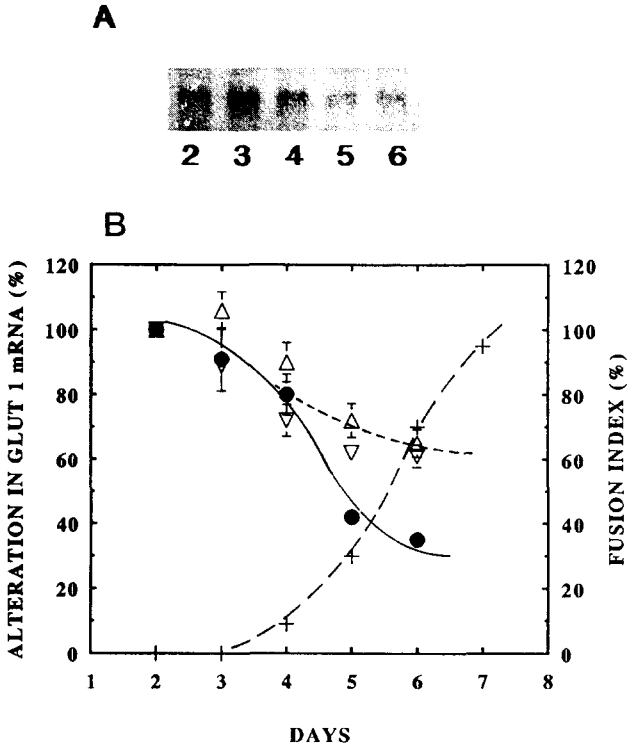

Fig. 1. Reduced levels of the GLUT 1 transcript during myogenesis and in confluent cultures. Poly(A)$^+$ RNAs were prepared from rat L6 myoblasts grown under different physiological conditions. Northern blot studies were carried out with labelled GLUT 1 and β_2-microglobulin cDNAs. Panel A is an autoradiogram showing the GLUT 1 transcript level of untreated L6 cells at different stages of myogenesis. Panel B shows the GLUT 1 transcript levels in myogenesis-competent and impaired cells. Rat L6 myoblasts were grown in the presence or absence of myogenesis inhibitors. The bands in the autoradiogram were quantified by using the JAVA Video Analysis Software, Jandel Scientific. The GLUT 1 transcript level in each culture was normalized according to the amount of the β_2-microglobulin mRNA present in each preparation. The GLUT 1 transcript level present in day 2 cultures was taken as 100%. ●, △, and ▽ denote the GLUT 1 transcript levels in untreated, phloretin (50 μM)- and BrdUrd (7.5 μM)-treated L6 myoblasts, respectively. This figure shows the result of three separate experiments using two separate mRNA preparations. Fusion indices (+) of untreated L6 cells were determined as previously described [44].

GLUT 1 transcript. In other words, GLUT 1 expression might be negatively regulated by the GLUT 4 isoform.

GLUT 1 expression was also examined in day 2 cultures seeded at different densities. When compared with cultures seeded with $0.2 \cdot 10^2$ cells/cm^2, about 26%, 50% and 60% reduction in the GLUT 1 transcript level were observed in day 2 cultures seeded with $0.3 \cdot 10^2$, $0.4 \cdot 10^2$ and $0.8 \cdot 10^2$ cells/cm^2, respectively. Thus, similar to GLUT 3 and 4 transcripts [1], GLUT 1 transcript level was reduced in confluent cultures. The reduction of all three GLUT transcripts indicated that a mechanism different from that associated with the above-mentioned GLUT 4 effect was probably involved.

3.2. GLUT 1 expression in GLUT 3⁻GLUT 4⁻ mutants

To further explore the relationship between the GLUT 1 and 4 expression, GLUT 1 transcript levels in several isogenic rat myoblast cell lines, L6, D23, M1 and M3, were examined. GLUT 1 transcript level in glucose-grown M1 and M3 mutants was twice that present in L6 cells (Table 1). Similar increase in the GLUT 1 transcript level was also observed in glucose-starved M1 and M3 mutants. It was important to note that such an increase was not observed with the parental GLUT 4^+ D23 cells. This suggested that the increase in the GLUT 1 transcript level was associated with the defect in the GLUT 4 isoform. This is the first genetic evidence indicating the inverse relationship between the expression of the GLUT 1 and 4 isoforms.

3.3. Positive regulation of GLUT 1 expression in rat myoblasts

We have previously demonstrated that glucose-starved (i.e., fructose-grown) myoblasts exhibit much higher glucose transport and CB-binding activities than their glucose-grown counterparts [15,40,41]. Since three different GLUT isoforms are present in rat L6 myoblasts [1], it is important to determine which GLUT isoform(s) are altered in glucose-starved cells. In this study, poly(A)$^+$ RNAs from day 2 cultures of glucose-grown and starved L6 myoblasts were probed with similar amounts of ^{32}P-labelled GLUT 1, 3, and 4 cDNAs (Fig. 2). Transcript levels were determined by the amount of cDNA bound to different amounts of the same transcript. Even though GLUT 1 was the least abundant GLUT transcript in glucose-grown cells (Fig. 2A), it was the most abundant one in glucose-starved cells (Fig. 2B). On the other hand, GLUT 3 and 4 transcript levels were not significantly altered by glucose starvation (Table 1). Thus, similar to multinucleated rat L6 myotubes [2,18,22], only the expression of the GLUT 1 isoform in mononucleated rat L6 myoblasts was positively regulated by events associated with glucose starvation.

To gain further insight into the regulatory mechanisms involved, the response of GLUT 4^- mutants to glucose starvation was examined. The GLUT 1 transcript levels in glucose-starved L6, D23, M1, and M3 cells were 217%, 175%, 173% and 184% of those present in their glucose-grown counterparts, respectively (Table 1). Thus, despite their defect in the GLUT 3 and/or GLUT 4 isoform, similar increases were observed in all the strains tested. If the GLUT cDNAs bind with their respective transcripts with similar efficiencies, a calculation based on data presented in Fig. 2B and Table 1 revealed that the GLUT 1 transcript level was about 1400% higher than the GLUT 3 and 4 transcript levels in glucose-starved M1 and M3 mutants. In other words, the GLUT 1 transcript is the predominant GLUT transcript present in these mutants.

3.4. Amount and subcellular distribution of the GLUT 1 transporter in various cell types

To examine the amount and subcellular distribution of the GLUT 1 transporter in various mutants, immunoblotting studies were carried out using saturating amount of the rabbit anti-GLUT 1 Ab (East Acres Biologicals, Southbridge, MA), and ^{125}I-protein A (Fig. 3, Table 2). Highly purified plasma membranes and microsomes were used in this investigation (see Methods and materials for properties). Since the GLUT 1 transporter level in D23 plasma membrane has been determined by CB binding studies [1,40], the GLUT 1 transporter content in other membrane preparations can be calculated by determining the amount of ^{125}I-protein A bound to the GLUT 1 immuno-complex associated with the D23 and other membrane preparations.

Table 1
Transcript levels of glucose transporters in glucose-grown and glucose-starved mutants

Carbon source	L6 (HAHT$^+$LAHT$^+$)	D23 (HAHT$^-$LAHT$^+$)	M1 (HAHT$^-$LAHT$^-$)	M3 (HAHT$^-$LAHT$^-$)
GLUT 1 transcript (2.8 kb)				
Glucose	100	113 ± 26.9	191 ± 11.1	196 ± 24
Fructose	217 ± 9.2	198 ± 34.5	331 ± 18.9	360 ± 42.1
GLUT 3 transcript (4.1 kb)				
Glucose	100	16 ± 4.0	12 ± 1.4	16 ± 3.5
Fructose	85 ± 5.6	11 ± 2.8	15 ± 8.7	15 ± 9.2
GLUT 4 transcript (2.8 kb)				
Glucose	100	107 ± 3.5	22 ± 8.4	28 ± 8.2
Fructose	102 ± 0	95 ± 3.2	22 ± 2.6	30 ± 7.2

Transcript levels for various glucose transporters were determined by Northern blot analysis. Poly(A)$^+$ RNAs were extracted from day 2 cultures of glucose-grown and fructose-grown (i.e., glucose-starved) L6, D23, M1, and M3 myoblasts. Labelled full length human erythrocyte GLUT 1, human fetal muscle GLUT 3, and rat insulin-responsive GLUT 4 cDNAs were used to detect the rat myoblast GLUT 1, GLUT 3 and GLUT 4 transcripts, respectively. The bands in the autoradiograms were quantified using the JAVA Video Analysis Software, Jandel Scientific. The GLUT transcripts in each culture were normalized according to the amount of β_2-microglobulin transcript present in each preparation. For each GLUT transcript, the level present in glucose-grown L6 cells was taken as 100%. Data indicate the results from three separate experiments. Transcript levels in glucose-grown L6, D23, and M1 cells were taken from Xia et al. [1], and were included for the ease of comparison.

GLUT 1 transporter levels in plasma membranes from glucose-grown cells were first examined (Fig. 3, Table 2). This transporter was present in the highly purified L6 plasma membrane at a concentration of 9.4 pmol per mg protein. The GLUT 1 transporter levels in D23 and M3 plasma membrane were 18.1 and 21.5 pmol per mg protein, respectively. A comparison with their respective

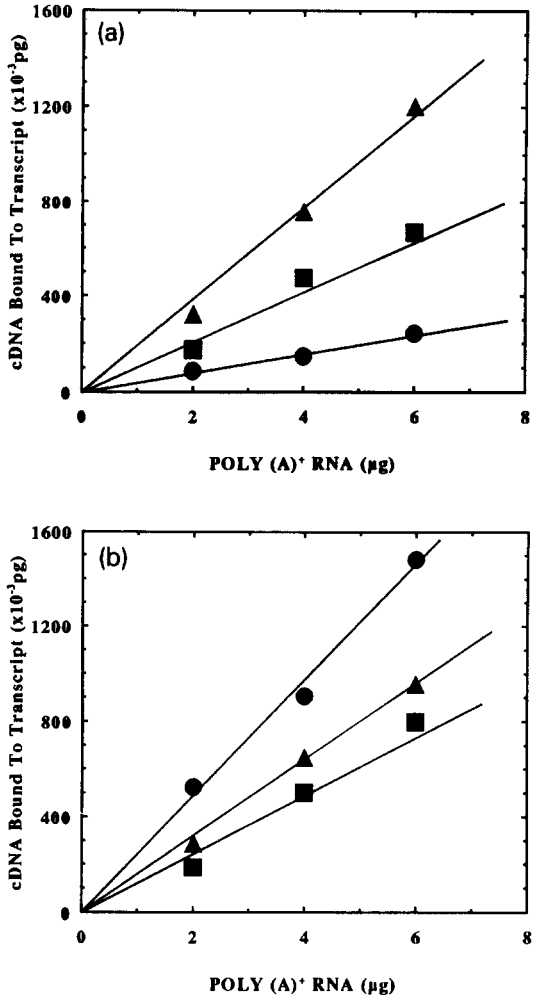

Fig. 2. Transcripts for glucose transporters in glucose-grown and -starved rat L6 myoblasts. Poly(A)$^+$ RNA was extracted from day 2 culture of rat L6 myoblast grown in the presence of 25 mM D-glucose or 25 mM fructose. Panels A and B show the GLUT 1, 3, and 4 transcript levels in glucose-grown and fructose-grown (i.e., glucose-starved) cells, respectively. Radioactive GLUT 1, 3, and 4 cDNAs were used to quantify the amount of specific transcripts in each RNA preparation. 40 ng of each type of cDNAs were labelled with [α-^{32}P]dCTP by random priming using the Prime-a-Gene kit. Hybridization of the blot was carried out with $2 \cdot 10^7$ cpm of the ^{32}P-labelled cDNAs. After washing, blots were exposed to Kodak XAR-5 films at $-70°$ C for 42 h. The autoradiogram was used to pinpoint the location of specific transcripts on the blot. After cutting out the spot containing the transcript, the amount of radioactivity associated with this spot was determined. Background counts were determined by the radioactivity associated with another spot of the same area on the same lane. After subtracting background counts, the amount of cDNA bound to a specific transcript was then calculated. ●, ▲, and ■ denote the amount of specific cDNA bound to the 2.8 kb GLUT 1, 4.1 kb GLUT 3 and 2.8 kb GLUT 4 transcripts, respectively.

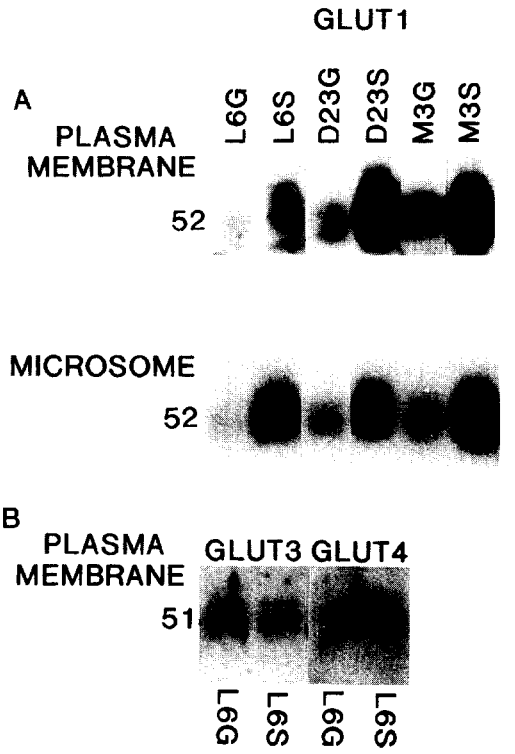

Fig. 3. Glucose transporter levels in plasma membranes and microsomes prepared from glucose-grown and starved myoblasts. Purified plasma membranes and microsomes were prepared from day 2 cultures of glucose-grown and starved myoblasts [38]. Electrophoresis and transfer onto nitrocellulose paper were performed as described in the text. Each lane was loaded with 60 μg protein. Nitrocellulose strips were incubated with antibodies raised the carboxyl termini of the rat GLUT 1 transporters, mouse GLUT 3, and rat GLUT 4 (East Acres Biologicals, Southbridge, MA). After washing, the strips were incubated for 4 h with 10^5 cpm of ^{125}I-protein A, and air-dried and exposed to X-ray film. Panel A shows the GLUT 1 transporter levels in the plasma membrane and microsomes prepared from glucose-grown and -starved L6 (L6G and L6S), D23 (D23G and D23S), and M3 (M3G and M3S) cells, respectively. Panel B shows the GLUT 3 and 4 transporter levels in plasma membranes prepared from glucose-grown and starved L6 myoblasts (L6G and L6S).

GLUT 1 transcript levels (Table 1) revealed that the GLUT 1 transporter content in these two mutants was considerably higher than expected. The observed increases were not due to subcellular redistribution of the GLUT 1 transporter, as similar increases were also observed in the microsomes (Table 2). This suggests that the translation and/or turnover rates of the GLUT 1 transporter are altered in the GLUT 3$^-$ mutants, D23 and M3.

GLUT 1 transporter levels in glucose-starved cells were also examined (Fig. 3, Table 2). When compared with their glucose-grown counterparts, plasma membranes from glucose-starved L6, D23, and M3 cells possessed an additional 26 pmol of the GLUT 1 transporter per mg protein; whereas microsomes from glucose-starved cells possessed an additional 13 pmol of the GLUT 1 transporter per mg protein (Table 2). Since similar increases were observed with all cell types, the glucose starvation response was not

influenced by the GLUT 3 or 4 isoforms. It should be noted that the GLUT 3 and 4 transporter levels in L6 cells were not altered by glucose starvation (Fig. 3).

3.5. Regulation of the functional states of the GLUT 1 transporter

Since GLUT 1 was the predominant GLUT transporter in mutants M1 and M3, these mutants could be used to examine the GLUT 1 transport properties free of interference by other GLUT isoforms. Analysis of transport data by Lineweaver-Burk plot clearly indicated that the absence of both GLUT 3 (K_m = 0.67 mM) and GLUT 4 (K_m = 3.5 mM) transport systems in the glucose-grown mutants (open symbols). The activities of the residual GLUT 3 and 4 transporters were too low to be detected. The line drawn by a linear least squares regression fit program extrapolated close to the origin of the plot; thus suggesting dGlc was taken up via a very low affinity transport process or via simple diffusion. Transport data were also analyzed by non-linear regression fit to the Michaelis-Menten hyperbola. The apparent K_m values for glucose-grown M1 and M3 mutants were found to be around 114 M and 369 mM, respectively. In other words, these glucose-grown mutants exhibited extremely low transport affinity for dGlc. In view of their very high plasma membrane GLUT 1 transporter content (21.5 pmol per mg protein) (Table 2), these studies indicated that the GLUT 1 transporter was not functional in glucose-grown cells.

In sharp contrast to glucose-grown cells, glucose-starved M1 and M3 mutants took up dGlc with an apparent K_m value of around 0.8 mM (solid symbols in Fig. 4). A non-linear regression fit revealed that the apparent K_m values for dGlc uptake by the glucose-starved M1 and M3 mutants were 1.07 ± 0.05 mM and 1.05 ± 0.16 mM, respectively. Since these mutants were devoid of the GLUT 2, 3 and 4 isoforms (Table 1 and [1]), the GLUT 1 transporter was likely responsible for this transport activity. After correcting for diffusion, the transport capacity of the GLUT 1 transport process in glucose-starved M3 cells

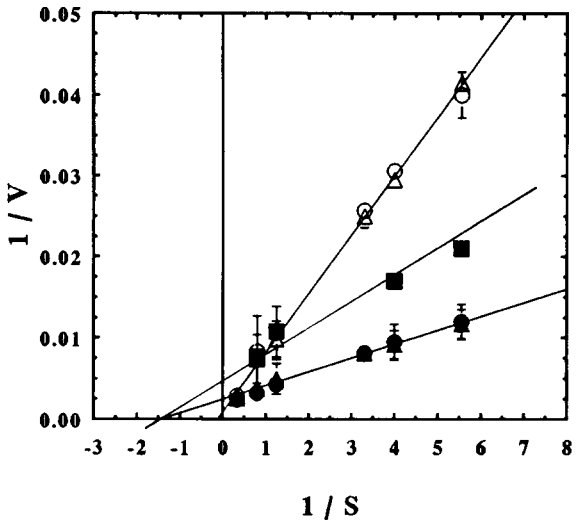

Fig. 4. Kinetics of 2-deoxy-D-glucose uptake by glucose-grown and starved M1 and M3 mutants. Transport studies were carried out with glucose-grown and starved M1 and M3 mutants as described in the text. Initial rates of uptake were calculated from the amount of dGlc taken up at 23° C in 15, 30, 45 and 60 s. The velocity (V) is expressed as pmol of dGlc taken up per min per 10^5 cells. S refers to the concentration of dGlc (in mmol per l) used. An equal amount of counts was added to each dGlc concentration. Data was analyzed by Lineweaver-Burk plot. ● and ▲ denote dGlc uptake by mutants M1 and M3, respectively. Open and solid symbols indicate uptake by glucose-grown and starved cells, respectively. The lines through the above symbols were drawn by a linear least squares regression fit program (SlideWrite Plus™, version 4.0, Advanced Graphics Software, Sunnyvale, CA). ■ indicates the net amount of dGlc taken up by the carrier-mediated transport process present in glucose-starved M3 cells; the amount of dGlc internalized via simple diffusion (as determined by the rates of dGlc uptake by CB-treated D23 cells) was subtracted from the raw data [1].

Table 2
GLUT 1 transporter levels in the plasma membrane and microsome of glucose-grown and starved mutants

		Plasma membrane			Microsome		
		glucose-grown cells	glucose-starved cells	starvation-stimulated increase	glucose-grown cells	glucose-starved cells	starvation-stimulated increase
L6	Protein A bound [a]	32.3	127.5	95.2	8.3	56.3	48.0
	CB bound [b]	9.4	37.0	27.6	2.4	16.4	14.0
D23	Protein A bound	62.3	163.3	101.0	26.7	71.2	44.5
	CB bound	18.1	47.4	29.3	7.7	20.7	13.0
M3	Protein A bound	74.0	151.7	77.7	28.8	75.5	46.7
	CB bound	21.5	44.0	22.5	8.4	21.9	13.5

[a] The amount of protein A bound is expressed as pg of protein A bound per mg protein.
[b] The amount of cytochalasin B (CB) bound is expressed as pmol of CB bound per mg protein.
Immunoblotting studies were carried out as described in Fig. 3. Saturating amounts of anti-GLUT 1 Ab and ^{125}I-protein A were used in these studies. The amount of ^{125}I-protein A associated with the GLUT 1 transporter immuno-complex was determined according to the method described in the text. Background counts were subtracted from each determination. Previous studies revealed that the molar content of the GLUT 1 transporter could be determined by the maximum binding capacity of the low affinity CB binding site (18.1 pmol per mg protein) in the glucose-grown D23 plasma membrane [40]. Knowing this value and the amount of ^{125}I-protein A bound to the glucose-grown D23 plasma membrane, GLUT 1 transporter levels in other membrane preparations were then calculated. Assuming one CB molecule will bind with one molecule of the GLUT 1 transporter, the amount of CB bound should reflect the number of GLUT 1 transporter present.

was calculated to be 220 pmol/10^5 cells per min (Fig. 4). Similar K_m value was also obtained when the data were fitted by non-linear regression. The protein content of rat myoblasts was previously shown to be around 0.55 mg/10^6 cells [13], and 10% of these was associated with the plasma membrane [38]. Based on these values, the GLUT 1 transport capacity of glucose-starved M3 cells was calculated to be 40 nmol per mg plasma membrane protein per min. This value was very similar to the GLUT 1 transporter level (44 nmol per mg plasma membrane protein) detected by immunoblotting studies with the glucose-starved M3 plasma membrane (Table 2). More importantly, these studies demonstrated the differences in the transport affinity of the GLUT 1 transporter present in glucose-grown and -starved cells.

3.6. Intrinsic property of the activated GLUT 1 transporter

The observation that the GLUT 1 transporter was the only functional GLUT transporter present in glucose-starved M1 and M3 mutants allowed an examination of the intrinsic property of this transporter without interference by other GLUT transporters. CB, phloretin, phlorizin and pCMBS are very potent inhibitors for various GLUT transporters [1]. While these inhibitors were previously shown to inhibit dGlc uptake by the glucose-grown M1 and M3 mutants by about 20% [1], they inhibited dGlc uptake by glucose-starved M1 and M3 mutants by about 60% (Table 3). It is therefore apparent that the intrinsic activity of the GLUT 1 transporter in glucose-starved mutants differ considerably from its glucose-grown counterpart.

The ability of the glucose-starved M1 and M3 mutants to take up MeGlc was also examined. Unlike their dGlc uptake properties, the GLUT 1 transporter present in these glucose-starved cells exhibited very low transport affinity for MeGlc (Fig. 5). Analysis by non-linear regression revealed that the apparent K_m values of the glucose-starved D23, M1 and M3 myoblasts were 4.4 ± 0.6 mM, 15.9 ± 1.7 mM and 12.8 ± 2.5 mM, respectively. Transport studies using much higher MeGlc concentrations also revealed that the apparent K_m value for MeGlc uptake was around

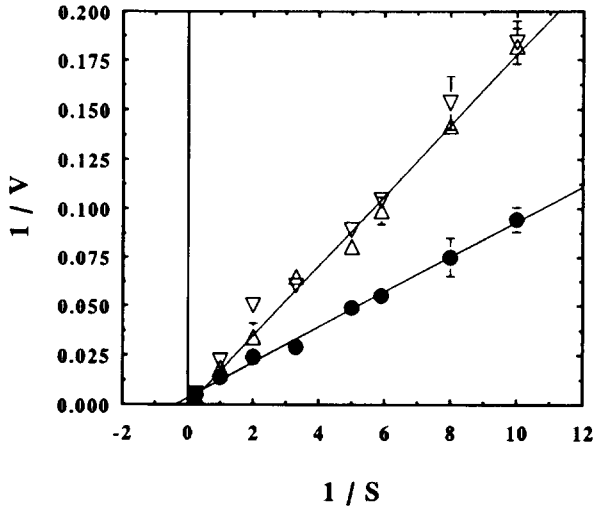

Fig. 5. Kinetics of 3-*O*-methylglucose uptake by glucose-starved M1 and M3 mutants and their parental D23 myoblast. Transport studies were carried out with glucose-starved cells as described in the text. Initial rates of uptake (V) were calculated from the amount of MeGlc taken up at 23° C in 15, 30, 45, and 60 s. The velocity is expressed as pmol of MeGlc taken up per min per 10^5 cells. S refers to the concentration of MeGlc (in mmoles per litre) used. An equal amount of counts was added to each MeGlc concentration. Data were analyzed by Lineweaver-Burk plot. ●, △, and ▽ denote uptake by glucose-starved D23, M1 and M3 cells, respectively. The lines through the symbols were drawn by a linear least squares regression fit program (SlideWrite Plus™, version 4.0, Advanced Graphics Software, Sunnyvale, CA).

10 mM (data not shown). Thus, similar to findings using the *Xenopus* oocyte expression system, MeGlc was a poor substrate for the GLUT 1 transporter [5].

The effect of D-galactose on dGlc uptake was also examined. Analysis of the data by Dixon plot revealed that D-galactose was a poor inhibitor for dGlc uptake by glucose-starved M1 and M3 cells, but not for L6 cells (Fig. 6). Inhibition of dGlc uptake by glucose-starved M1 and M3 cells could not be observed when transport assays were carried in the presence of 3 mM D-galactose. Thus, unlike the GLUT 3 and GLUT 4 transport processes [31], D-galactose was a poor substrate for the rat myoblast GLUT 1 transporter. Studies with the *Xenopus* oocyte

Table 3
Effects of specific hexose transport inhibitors on dGlc uptake by rat myoblast mutants

Cultures	Glucose transporter isoforms	dGlc uptake by untreated cells (pmol/10^5 cells per min)	Cytochalasin B (3 μM)	Phloretin (10 μM)	Phlorizin (30 μM)	pCMBS (2 mM)
Glucose-grown L6	GLUT 3/4	11.42 (100%)	26%	33%	61%	43%
Glucose-grown D23	GLUT 4	4.22 (100%)	0%	0%	5%	10%
Glucose-starved M1	GLUT 1	18.52 (100%)	50%	52%	65%	50%
Glucose-starved M3	GLUT 1	15.72 (100%)	36%	44%	57%	37%

Day 2 subconfluent cultures were used in these studies. Transport studies were carried out as described in the text. The concentration of dGlc used was 0.06 mM. Inhibitors were added at time zero. Samples were taken at 15, 30, 45, and 60 s. The amount of dGlc internalized via simple diffusion (as determined by the rate of dGlc uptake by the cytochalasin B-treated D23) was subtracted from the raw data [1]. Results were expressed as percentages of untreated cells. Data were the means of three independent experiments, with an average error of 5%.

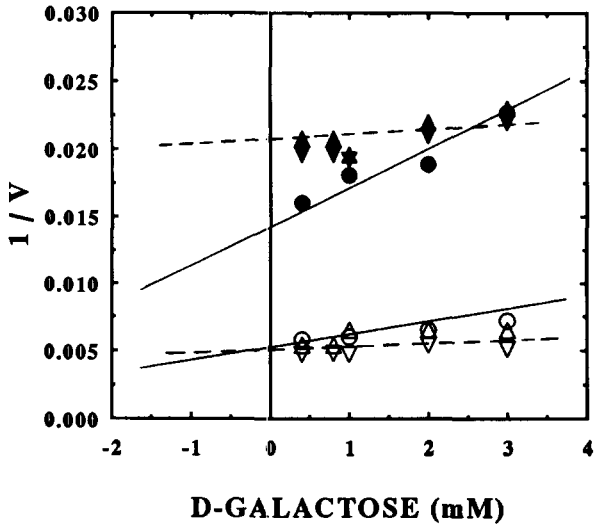

Fig. 6. Effect of D-galactose on 2-deoxy-D-glucose uptake by glucose-starved M1 and M3 mutants. Transport studies were carried out as described in the text. Initial rates of uptake (V) were calculated from the amount of dGlc taken up at 23° C in 15, 30, 45, and 60 s. The velocity (V) is expressed as pmol of dGlc taken per min per $1 \cdot 10^5$ cells. The effect of D-galactose on dGlc uptake by glucose-starved L6 (●), M1 (▲) and M3 (▼) mutants was analyzed by Dixon plot. The filled and open symbols denote uptake of 0.1 mM and 0.4 mM dGlc, respectively. The K_i value of D-galactose on dGlc uptake by L6 myoblasts was 4.5 mM.

expression system also indicated that D-galactose was a poor substrate for the GLUT 1 transporter [5].

4. Discussion

The regulation and properties of glucose transporters (GLUT) have been examined in a number of cell types [2–7,50,62–67]. At least seven different GLUT isoforms have been identified. The fact that two or more of these isoforms are found in each cell type [6] makes it difficult to determine the identity, regulation, property and functional states of a specific GLUT transporter. The present investigation examined the expression and intrinsic property of the GLUT 1 isoform using two independent spontaneous rat myoblast mutants, M1 and M3. The following findings indicate that these mutants are ideally suited for these studies. (i) Both mutants possessed very high levels of the GLUT 1 transcript and transporter (Tables 1 and 2, Fig. 3). (ii) They were devoid of the GLUT 2 isoform [1]. (iii) They possessed less than 15% of the L6 GLUT 3 transcript and transporter (Table 1, [1]), and were devoid of the GLUT 3 transport activity (Fig. 4). (iii) They exhibited no detectable GLUT 4 transport activity (Figs. 4 and 5), even though they harboured about 24% of the L6 GLUT 4 transcript and transporter levels (Table 1, [1]). (iv) The absence of the GLUT 3 and 4 transport processes in these mutants was corroborated by studies using potent glucose transport inhibitors and sugar analogues ([1], Fig. 6). (v) The GLUT 5 isoform was not likely responsible for the CB-sensitive dGlc transport activity observed in the glucose-starved M1 and M3 mutants (Fig. 4 and Table 3). This was because the GLUT 5 transporter could not take up dGlc and was insensitive to CB treatment [5]. (vi) Finally, the GLUT 1 transcript level in the glucose-starved M1 and M3 mutants was 14-times higher than those of GLUT 3 and 4 (Table 1 and Fig. 2B). It may be apparent that these mutants can serve as unique tools for examining the in situ expression, and property of the GLUT 1 isoform without interference by other GLUT isoforms.

GLUT 1 expression is subject to different types of negative regulation. First, GLUT 1 expression is influenced by the presence of the GLUT 4 isoform. GLUT 4^- mutants possessed twice the amount of GLUT 1 transcript present in the isogenic GLUT 4^+ L6 and D23 myoblasts (Table 1). The GLUT 1 transporter content in the GLUT 4^- plasma membranes and microsomes was also higher than that in D23 and L6 myoblasts (Table 2 and Fig. 3). Since M1 and M3 are two independent spontaneous mutants selected from D23 myoblasts, the increase in GLUT 1 expression is probably associated with the absence of the GLUT 4 isoform in these two mutants. The inverse relationship between the GLUT 1 and 4 isoforms was also observed in myogenesis-competent and -impaired L6 myoblasts. For example, the L6 myotube GLUT 4 and GLUT 1 transcripts were 7500% and 25% of the respective day 2 levels (Fig. 1 and [1]). Inhibition of myogenesis resulted in a reduced increase in GLUT 4 transcript and a reduced decline in the GLUT 1 transcript (Fig. 1 and [1]). The inverse relationship between these two isoforms was also observed in other cell types undergoing developmental and physiological changes [55,68–70] or treated with insulin, cAMP, tumor necrosis factor or monokines [61,71–73]. An alternative explanation is that both M1 and M3 mutants are defective in a regulatory element, tentatively referred to as the R factor, which can regulate the expression of the GLUT 1 and 4 isoforms in opposite ways. We are currently examining this possibility by transfecting the M3 mutant with the GLUT 4 cDNA.

Second, GLUT 1 expression was reduced during myogenic differentiation (Fig. 1). The GLUT 1 transcript level in multinucleated myotubes (day 6 culture) was about 25% of that present in day 2 culture; the major decline occurred only upon the appearance of myotubes (i.e., between days 4 and 5) (Fig. 1). This dramatic reduction was not observed in cells impaired in myogenesis; 40% and 70% of the day 2 GLUT 1 transcript level were detected in day 5 culture of myogenesis-competent and -impaired cells, respectively. Two lines of evidence indicate that the GLUT 1 transcript level is altered as a result of changes in the GLUT 4 isoform, and not a direct result of myogenesis. (i) During growth, a 4-fold increase in the GLUT 4 transcript (day 3.5) was detected before the major decline of the GLUT 1 transcript (i.e., between day 4 and 5) (Fig. 1, [1]). Similar temporal order of expression was also observed in adipose tissues [61]. (ii) While the level of the GLUT 1

isoform differed considerably between the GLUT 4^- mutants (M1 and M3) and their parental GLUT 4^+ D23 cells, all three clones were impaired in myogenesis [44]. This clearly indicated the difference in GLUT 1 isoform was independent of the cells' ability to undergo myogenesis. Thus, while GLUT 4 expression is regulated during myogenesis, GLUT 1 expression is directly affected by GLUT 4.

Third, immunoblotting studies revealed that the GLUT 1 transporter level in the plasma membrane and microsomes of the GLUT 3^- myoblasts (D23 and M3) was higher than predicted by their GLUT 1 transcript level (Table 1 and 2; Fig. 3). This suggested that the translation and/or stability of the GLUT 1 transporter was affected by the GLUT 3 isoform. Fourth, GLUT 1 expression was negatively regulated in confluent cultures. When compared with cultures seeded at $0.2 \cdot 10^2$ cells/cm^2, about 60% reduction in the GLUT 1 transcript level was observed in day 2 cultures seeded at $0.8 \cdot 10^2$ cells/cm^2.

GLUT 1 expression is subject to positive regulation in glucose-starved myoblasts. First, both GLUT 1 transcript and transporter levels, but not those of the GLUT 3 and 4 isoforms, were increased by about 2-fold in glucose-starved myoblasts (Tables 1 and 2; Fig. 3). This was similar to L6 multinucleated myotubes [2,18,22] and rat kidney cells [74]. Mutant studies indicated that this glucose starvation-mediated increase was mediated by processes independent of the GLUT 3 and 4 isoforms (Tables 1 and 2). Thus, the synthesis and/or stability of the GLUT 1 isoform are positively regulated in glucose-starved cells.

Second, an examination of the GLUT 1 transport affinities and responses to inhibitors revealed that the intrinsic activity of this transporter was dramatically altered in glucose-starved cells. Analysis of transport data by linear and non-linear regression programs revealed that dGlc was taken up by glucose-grown M1 and M3 myoblasts either via an extremely inefficient transporter, or via simple diffusion (Fig. 4), despite the relatively high GLUT 1 transporter level (22 pmol/mg protein) detected in their plasma membrane (Table 2). In contrast, the GLUT 1 transporter in glucose-starved cells took up dGlc with an apparent K_m value of around 0.9 mM (Fig. 4). This transporter also differed from its glucose-grown counterpart in its responses to cytochalasin B, phloretin, phlorizin, and pCMBS (Table 3). This indicated that the functional state and intrinsic property of the GLUT 1 transporter were altered by events associated with glucose starvation. Similar to findings using the *Xenopus* oocyte expression system [5], both MeGlc and D-galactose were relatively poor substrates for the GLUT 1 transporter present in glucose-starved myoblasts (Figs. 5 and 6).

The GLUT 1 intrinsic activity in other cell types was also affected by its immediate environment, and was activated under a number of conditions. For example, even though 3T3-L1 adipocytes contained 30 fold more cell surface GLUT 1 transporter than CHO cells, they exhibited only 41% of the CHO GLUT 1 transport activity [19]. Human GLUT 1 expressed in 3T3-L1 cells also had a lower intrinsic activity than when expressed in CHO cells [75]. The GLUT 1 intrinsic activity in 3T3-L1 fibroblasts was significantly reduced upon differentiation into adipocytes, even though the latter possessed 2.6-times more cell surface GLUT 1 transporter. Activation of dGlc uptake by glucose-grown 3T3-L1 adipocytes, rat adipocytes and clone 9 cells by cholera toxin, protein synthesis inhibitors, energy poisons and cadmium was found to be mediated by changes in the GLUT 1 intrinsic activity, without increasing the level of the cell surface GLUT 1 transporter [3,75–78]. The GLUT 1 transporter was probably catalytically inert under basal conditions, even though it was immunoactive in the plasma membrane. These cryptic transporters could be activated by mechanisms involving protein-protein interaction, covalent or noncovalent modifications of transporters, or changes in their microenvironment.

The rat myoblast GLUT 1 transporter in glucose-grown cells may also exist in a cryptic form, which can be activated by events associated with glucose starvation. Increases in basal dGlc uptake activity were also observed when glucose-grown myoblasts were treated with 2,4-dinitrophenol for 24 h [31]; however, increases in transport activity could not be detected when glucose-starved cells were used instead. In other words, further activation could not be achieved once the GLUT 1 transporter has been activated by glucose starvation.

In summary, this study showed that the expression of the rat myoblast GLUT 1 transporter was subject to different types of regulation. Studies with mutants defective in GLUT 3 and/or GLUT 4 provided valuable information not only on the in situ regulation of GLUT 1 expression, but also on the intrinsic property of the GLUT 1 transporter.

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